2016 년 8 월

석사학위 논문

Analysis of Cyanide in Cigarette Smoke and Characterization of Detoxification Process in Oral Cavity

조선대학교 대학원

치의 학 과

Vu Phuong Dong

Analysis of Cyanide in Cigarette Smoke and Characterization of Detoxification Process in Oral Cavity

담배연기의 시아나이드 성분 분석 및 구강내해독기전 연구

2016 년 8 월 25 일

조선대학교 대학원

치의 학 과

Vu Phuong Dong

Analysis of Cyanide in Cigarette Smoke and Characterization of Detoxification Process in Oral Cavity

지도교수 유 훈

이 논문을 치의학 석사학위신청 논문으로 제출함

2016 년 4 월

조선대학교 대학원

치의 학 과

Vu Phuong Dong

Vu Phuong Dong 의 석사학위논문을

위원장 조선대학교 교 수 **김 명 수** (인)

위 원 조선대학교 교 수 **방 일 수** (인)

위 원 조선대학교 교 수 **유 훈** (인)

2016 년 5 월

조선대학교 대학원

Table of contents

List of figures

List of tables

ABSTRACT

Analysis of Cyanide in Cigarette Smoke and Characterization of Detoxification Process in Oral Cavity

Vu Phuong Dong

Advisor: Prof. Hoon Yoo, Ph.D.

Department of Bio-dental Engineering,

Graduate School of Chosun University

Cigarette smoke contains high concentration of poisonous hydrogen cyanide (HCN) gas which is easily absorbed in human body. In this thesis, we developed a capillary electrophoresis (CE) method to analyze cyanide (CN) component in cigarette smoke and understand how oral cavity response to the CN substances. CN was extracted by bubbling cigarette smoke through various isotonic solutions at various pH conditions. The concentration of CN in the solutions was determined by the oxidation reaction of hydrogen peroxide, converting CN into thiocyanate ion (SCN^-), prior to the detection by CE. Interestingly, CN^- ion is biologically detoxified into SCN^- by the action of sulfur transferase enzyme, rhodanese. In the present study, all of cell lysates prepared from human gingiva cells (YD-38), human tongue cells (YD-15) and human epithelial cells (KB and fadu) converted CN^- into SCN^-. Among cell lysates, YD-38 cells showed the highest conversion rate. RT-PCR confirmed the expression of rhodanese gene in all oral cancer cells investigated. The secretion of SCN^- from the cells was confirmed by

CE, supporting that CN uptaken in oral cavity cells was not only converted into SCN^- but also secreted out into saliva.

국문초록

담배연기의 시아나이드 성분 분석 및 구강내해독기전 연구

Vu Phuong Dong

지도교수 유훈

치의 학과

조선대학교 대학원

독성을 지니는 hydrogen cyanide(HCN) 가스는 담배연기의 성분이며 인체에 CN^-의 형태로 흡수되어 독성을 유발한다. 체내에서 cyanide 는 sulfur transferase 효소인 rhodanese 에 의해 thiocyanate (SCN^-)로 전환되는데 이로 인해 담배를 많이 피우는 사람의 혈청 또는 체액에는 SCN^-이 더 높은 농도로 검출되는 것으로 보고되었다. 그러나 구강세포 내에서 SCN^- 형성 및 축적기전은 아직 명확히 밝혀진 바 없다. 본 연구에서는 전기영동 장비를 이용하여 담배연기 내의 CN^- 성분을 분석하고 CN^- 성분이 구강 내에서 어떻게 비독성 성분으로 전환되는 지를 조사하였다. 담배연기 성분인 HCN 은 다양한 pH 의 isotonic 용액에서 포집하였다. 용액 내의 HCN 농도를 평가하기 위해서 HCN 과 thiosulfate 를 과산화수소 산화반응에 의해 SCN^-으로 전환시킨 후 전기영동 장비 (capillary electrophoresis, CE)를 이용하여 SCN^-을 검출하였다. 조사한 human 잇몸세포 (YD-38), 혀 세포 (YD-15), 상피세포 (KB 와 Fadu)의 lysate 에서 CN^-이 SCN^-으로 전환된는 것을 확인하였다. 특히 YD-38 세포에서 상대적으로 큰 전환을 보였다. 또한 구강세포들이 rhodanese 유전자를 발현하고 있음을 RT-PCR 을 이용해 확인하였다. 마지막으로, CN^-에 노출시킨 세포를 배양한 배지에서 SCN^-을 전기영동에 의해 검출함으로써 구강 내에서 세포로 유입된 CN^- 이온이 rhodanese 에 의해 SCN^-으로 전환된 후 타액으로 분비될 수 있음을 확인하였다.

1. Introduction

Cyanide (CN) is one of the most toxic poisons. It exists as a colorless gas (HCN), inorganic solid salts (sodium cyanide or potassium cyanide) or organic compounds (nitriles). Most of these are highly toxic. Especially, HCN is dangerous toxic gas with high lethality [1]. The physical properties of HCN are summarized in Tab. 1.

Table 1. Physical properties of hydrogen cyanide

Description	Colorless liquid/gas
Molecular formula	HCN
Molecular weight	27.03
Boiling point	25.6 ^{o}C
Melting point	-13.4 ^{o}C
Vapor pressure	0.83 atm at 20 ^{o}C
Solubility	Miscible in water, alcohol; slightly soluble in ether

Tobacco cigarette smoke (CS) contains high level of HCN which is generated during the combustion of the proteins or nitrate compounds at high temperatures under the oxygen deficient condition [2]. HCN level in inhaled CS ranges from 10 to 400 μg per cigarette (U.S. brands) and 0.6 to 27 % of CS (by weight) are found in secondary smoke [3]. Although no one wants to breathe HCN into their lung, smokers do it multiple times while smoking. The CN^- ion acts as a non-competitive inhibitor to cytochrome c oxidase in mitochondria by complexing to the iron within this enzyme. The binding of CN prevents transport of electrons from cytochrome c to oxygen, thus disrupts the electron transport chain and energy production [1]. World health

organization (WHO) reported that chronic cyanide exposure by smoking causes neurological, respiratory, cardiovascular and thyroid problems [4-5]. With the rapid increase in the number of smokers, the concerns on the toxic effects of cyanide are increased [6].

In humans, several biological pathways prevent the toxic effects of CN. These pathways involve the conversions of cystine into aminothiazoline- and iminothiazolidine carboxylic acids, alpha ketoglutarate into glutarate cyanohydrin and hydroxycobalamin (vitamin B12a) into cyanocobalamin (vitamin B12). Approximately 80 % of CN is metabolized to SCN by the action of mitochondrial sulfur transferase enzyme called rhodanese (Rh) (Fig. 1) [7].

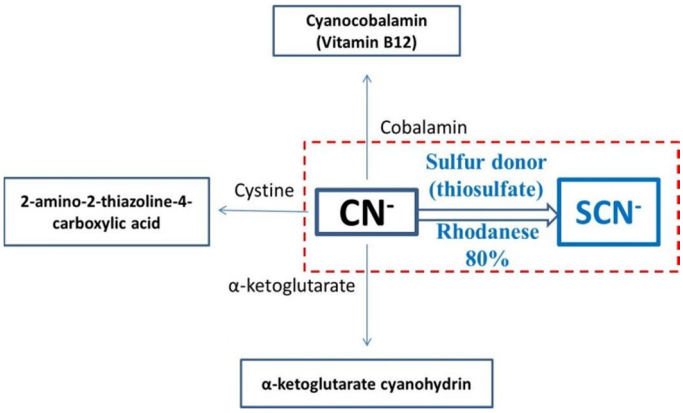


Figure 1. Scheme of cyanide detoxification in human

Rhodanese is known to be a multifunctional enzyme. It was reported to be involved in the formation of iron–sulfur center in proteins [8-10], participation in energy metabolism [11-12], functioning as a thioredoxin oxidase [13] and metabolizing CN [14-

15]. The activity of rhodanese in the tissues is associated with the CN detoxification [16].

Thiosulfate ion ($S_2O_3^{2-}$) has been widely used as sulfur donor in many assays to demonstrate rhodanese activity from biological samples. The conversion reaction of CN to SCN using $S_2O_3^{2-}$ is shown in Eq. 1.

(1)

The conversion rate was estimated by analyzing SCN^- produced. To do this, Sorbo assay, one of the most prominent assays, was done by the determination of the deep red ferric thiocyanate ($Fe(SCN)_3$) complex, which formed from the reaction between SCN^- and ferric ion at 464 nm [17-18]. Although this is a simple, fast and suitable assay, the competitive effects of other metal ions with iron in complex formation as well as the overlapping absorbance of other colored components interrupt the accurate measurement. To overcome this limitation, we used a capillary electrophoresis (CE) to directly detect SCN^- from reaction mixture. Previously, SCN^- could be detected by ion chromatography (IC). However, in comparison with IC, CE has more advantages in terms of the sample volume, detection limit, resolution and analysis time [19]. CE also overcomes the limits of Sorbo assay since it detects SCN directly.

In human, high level of Rh was found in kidney and liver tissues [20], suggesting CN to SCN conversion mainly occurs in these organs. The SCN is then released into the serum, increasing serum level of SCN^-. Interestingly, SCN concentration in human oral and nasal secretions is even much higher than serum.

Concentration of SCN in saliva was at 0.5 to 3 mM which is more than ten folds higher than serum (0.05 mM) (Tab. 2).

Table 2. SCN level in human saliva, nasal discharge and serum

Compartment	[SCN] (μM)	References
Saliva	500-3000	[21-22]
Nasal airway fluid	100-1200	[23]
Serum	5-50	[23-24]

Oral cavity is the first organ exposed to CN in cigarette smoke. In this study, we hypothesized that the inherent conversion of CN into SCN would be occurred at oral environment. The specific goals were to characterize CN detoxification in oral and nasal cavities *in vitro* and understand the reason of high level of SCN^- in saliva. The experiments were designed to detect CN in cigarette smoke and CN to SCN conversion in oral, nasal discharge and oral cells (Tab. 3). The expression of rhodanese in the oral cells was determined by reverse transcription polymerase chain reaction (RT-PCR). The CN to SCN conversion by cell lysates and SCN secreted out from CN-exposed oral cancer cells was detected by CE.

Table 3. Experimental methods

Specimens	**Cigarette smoke**	**Oral cells** (YD15, YD38, KB & fadu)	**Fluids** (Saliva, nasal discharge)
Conversion process	**HCN**	**Rhodanese** →	**SCN**
Methods	• Chemical reaction • CE detection	• RT-PCR • Lysate experiments • CN exposure tests	• Colorimetric assays • CE detection

2. Materials and Methods

2.1. Chemicals

NaCN, $Na_2S_2O_3$ and KSCN were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Other chemical reagents of analytical grade were supplied by commercial sources. CE background electrolyte (BGE) and standard solutions were prepared with Milli-Q water (18mΩ cm) produced by a purification system (Millipore, Molsheim, France). The BGE was 25 mM phosphate buffer and 1 mM myristyltrimethyl ammonium hydroxide (Waters, Mapple, Milford, USA) at pH 7.0. Fresh BGE was daily prepared, membrane filtered 0.45 μm (Millipore) and degassed prior to use.

2.2. Capillary electrophoresis

The analysis procedure was performed on a Beckman Coulter P/ACE MDQ Capillary Electrophoresis System and interfaced with the 32-Karat software (Beckman, Kraemer, CA). Fused silica capillaries (100 μm i.d. and 365 μm o.d.) were supplied from Polymicro Technologies (Phoenix, AZ, USA) with the total length and effective length were 60 cm and 50 cm, respectively. The detection was set at a wavelength of 200 nm, using a mercury lamp. New capillaries were rinsed under the 20 psi (1 psi = 6894.76 Pa) pressure with the following steps: 100 % MeOH for 5 min; 0.1 mol/l HCl for 5 min; 0.1mol/l NaOH for 5 min; distilled water for 3 min and BGE for 3 min. Between injections, the capillary was reconditioned by rinsing with 0.1 mol/l HCl for 1 min, 0.1 mol/l NaOH for 1 min and BGE for 1 min. Electrophoresis was carried out at 20 kV with positive polarity. Samples were introduced hydrodynamically with the injection

time of 3 s at an over pressure of 0.5 psi. The electrophoretic procedures were conducted at 25^0C.

2.3. Quantification of cyanide in cigarette smoke

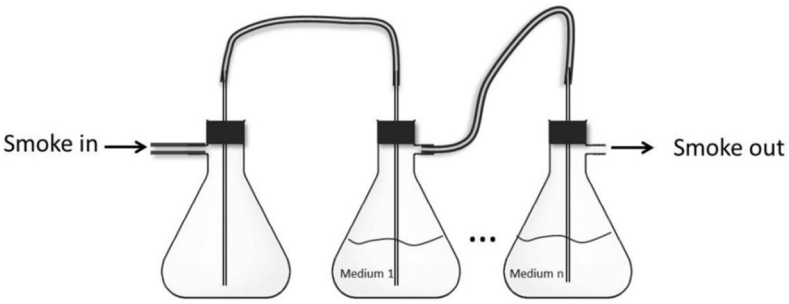


Figure 2. The extraction of HCN from smoke

Cigarette smoke extract (CSE) was prepared by bubbling smoke from one cigarette in 40 ml of solution by using a smoke extractor (Fig. 2). To determine the concentration of CN, 100 µL of smoke extract was mixed with the same volume of 5 mM of H_2O_2 and 5 mM of $Na_2S_2O_3$, incubated at 50^0C for 30 minutes. Then SCN generated was analyzed by CE. Since the mole ratio of SCN and CN in the chemical reaction is 1 to 1, the determined value of SCN concentration was considered as the concentration of CN in CSE.

2.4. Sample preparation from saliva and nasal discharge

Saliva and nasal discharge were collected into the plastic tubes. All volunteers were demanded to rinse their mouth with distilled water prior to the collection of saliva. Nasal discharges were collected from people who had cold or allergic rhinitis. The preparation procedure was as follows: (i) collecting samples; (ii) weighting, (iii)

centrifuging (10.000 rpm for 10 min) and (iv) collecting supernatants and keeping at -20^{0}C. Exactly 100 µg of supernatant of each sample was used for checking rhodanese activity.

2.5. Cell preparation

All cell lines used in this study were purchased from Korean cell bank (Korea). Phenol red-free RPMI 1640 medium was used for culturing YD-15 and YD-38 cell lines. KB cells were grown in Dulbecco's modified eagle's medium (DMEM) and Fadu cells were cultured in Minimum essential medium (MEM). All media were supplemented with 10 % FBS and antibiotics (100 U/ml penicillin G and 100 µg/ml streptomycin sulfate). Cells were seeded under humidified atmosphere, 5 % CO_2/95 % air at 37 ^{0}C in a incubator. To prepare the lysates, cells were washed twice with Hanks' Balanced Salt Solution (HBSS), scrapped and ultrasonicated for 5 min after diluting by 500 µL distilled water. After that, the mixture was centrifuged at 13.000 rpm for 15 min at cold temperature (4 ^{0}C) and the supernatant was collected. The concentration of cytoplasmic protein was determined by Bradford method [25] using reagents from the Bio-Rad DC protein micro-assay kit (Bio-Rad Laboratories, Hempsted, UK). The lysate samples were diluted into protein concentration of 800 and 400 µg/ml for determination of rhodanese activity.

2.6. Detection of rhodanese activity

Rhodanese activity was determined by using either Sorbo colorimetric assay or CE. The applied colorimetric assay was based on the report of Agboola et al. [26]. 20 µl of sample (fluids or cell lysates) was mixed 40 µl of 250 mM of $Na_2S_2O_3$, 40 µl of 250 mM

NaCN, 100 μl of 1 mM tris-(hydroxymethyl)-aminomethane hydrochloride buffer, pH 8.0. The mixture was incubated at 37°C for 3 min and then quenched by adding 200 μl of 4 % of formaldehyde. SCN produced from reaction was reacted with 0.6 ml of Sorbo reagent (consisting a ferric nitrate solution containing 0.025 g of $Fe(NO_3)_3.9 H_2O$ in 0.74 ml water and 0.26 ml concentrated nitric acid) to form the red colored complex which can measured at 464 nm by an ELISA plate reader (Multiskan EX, Thermo Electron corporation). For CE, SCN^- samples were 10 folds diluted and filtered (0.45μm) prior to injection. CE conditions were described in section 2.2. Sodium urate and distilled water were used as an internal standard and control, respectively. Each experiment was repeated more than three times.

2.7. RT-PCR

Total RNA was extracted from all tested cell lines (YD-15, YD-38, KB and fadu cells) by using RNeasy Mini Kit (QIAGEN, USA). Contaminated genomic DNA in total RNA was removed by RNase-free DNase I digestion. Rhodanese oligonucleotide primers were designed from conserved domains of Rh exon 2 and exon 3 sequences. The sequence of Homo sapiens Rh, RefSeqGene on chromosome 22 (Genbank accession number NG_027994.1) was used as a reference sequence. PCR conditions were designed by using Primer3 software [27] and Oligo calc webtool [28]. Sequences of primers were listed in Table 4. The reverse transcription was performed in a total volume of 50 μl containing 1 μg of total RNA, $Oligo(dT)_{12-18}$ Primer and SuperScript™ II Reverse Transcriptase (Invitrogen, USA), using 2720 Thermal cycle (Life technology™). Each PCR was run in triplicate using Hotstart PCR premix (Bioneer), which contains 1 μl of cDNA template and primers to be the final volume of 20 μl.

Program of RT-PCRs were 35 cycles of 30 seconds at 95 ^{0}C, 52 ^{0}C and 72 ^{0}C, completed by extension at 72 ^{0}C for 7 minutes (10 minutes for Rh exon 2-3). RT-PCR products were separated on 1.2 % agarose gel in TBE, stained with ethidium bromide and detected by UV-lamp at 302 nm. Glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene was used as a reference.

Table 4. Primer sequence for RT-PCR

Primer	Sequence (5'-3')	Product (bp)
h-GAPDH (F)	CTC TGA CTT CAA CAG CGA CA	200
h-GAPDH (R)	TCT CTC TCT TCC TCT TGT GC	
Exon 2F	TGG TTC ATC AGG TGC TCT AC	520
Exon 2R	GAT TCA AGG TTC TCC AGC AC	
Exon 3F	TCA TGG ACT TCC TGA CTG AG	347
Exon 3R	TCA TCT CCT TCA CCT AAG AG	

2.8. Cyanide exposure on cells

YD-38 cells (1×10^6 cells/ml) were cultured in 100 mm dishes with RPMI modified medium that contains 25mM of HEPES hemi-sodium salt for 24 h prior to treat compounds. After treating mixture of NaCN and $Na_2S_2O_3$ at concentration of 25 mM, the cell morphology was observed by an Axiovert microscope (Carl Zeiss). Every 3 hours, 100 μl of culture medium was collected and qualified the level of SCN^- by CE. The cells with only $Na_2S_2O_3$ treatment were used as a control.

3. Results

3.1. Optimization of the capillary electrophoresis conditions

In the enzyme conversion reaction (Eq. 1), cyanide and thiosulfate are starting materials while thiocyanate and sulfite are products. Therefore, analyzing the composition of the mixture of these anions is important to measure of enzyme activity. The influence of background electrode (BGE) pH on the CE peaks detection was examined in the pH range 5.0–8.5 of sodium phosphate at concentration of 25 mM. The BGE conditions at lower than pH 5 were not used since analysts were not separated. The BGE conditions at higher than pH 8.5 caused boiling of the electrolyte and poor reproductions. Table 5 summarizes the resolution and RSD of detected peaks. Among the tested pH conditions, the condition at pH 7.0 offered the most acceptable resolution (R = 1.28), best repeatability for migration time (0.34 %), peak area (0.91 %) and peak height (0.82 %). Showing in the Fig. 3 is overlapping of three independent CE runs obtained by using buffer consists of 25 mM phosphate and 1 mM of OFM at pH 7.0. The optimum condition was used to make the calibration curve of SCN^- as well as selected for further experiments in this study.

The calibration graph in Fig. 4 was obtained by the analytical procedure that was carried out on the standard solution containing 0.2 to 1.0 mM of SCN^-. The peak height and peak area were plotted against the amount of SCN^- and the linear calibration was obtained after 10 runs. The correlation coefficients were better than 0.99 in both cases. The linearity equations are both acceptable for the calculation of SCN^- concentration.

Table 5. Effect of pH on CE resolution and repeatability.

pH of BGE	Mean of peak resolution ($SCN^-/S_2O_3^{2-}$)	RSD of SCN peak (%) (n=5)		
		Migration time	Peak area	Peak height
5.0	1.01	1.68	3.12	2.79
6.0	1.08	3.14	2.54	5.64
7.0	**1.28**	**0.34**	**0.91**	**0.82**
8.5	1.47	3.72	6.38	7.99

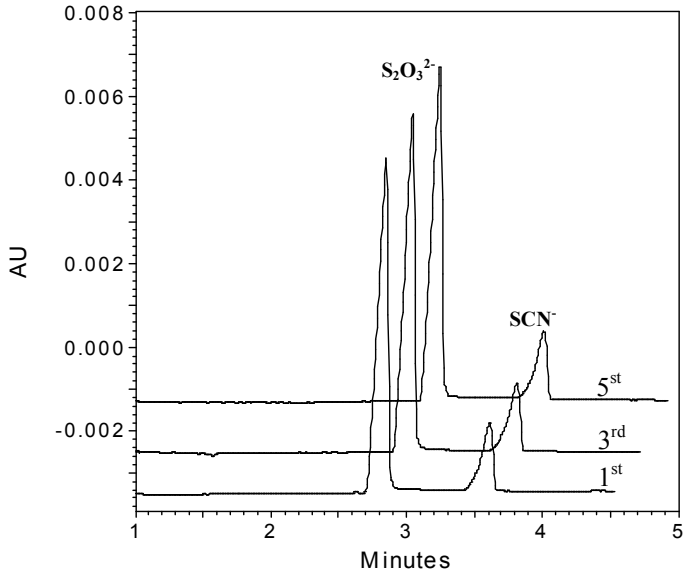
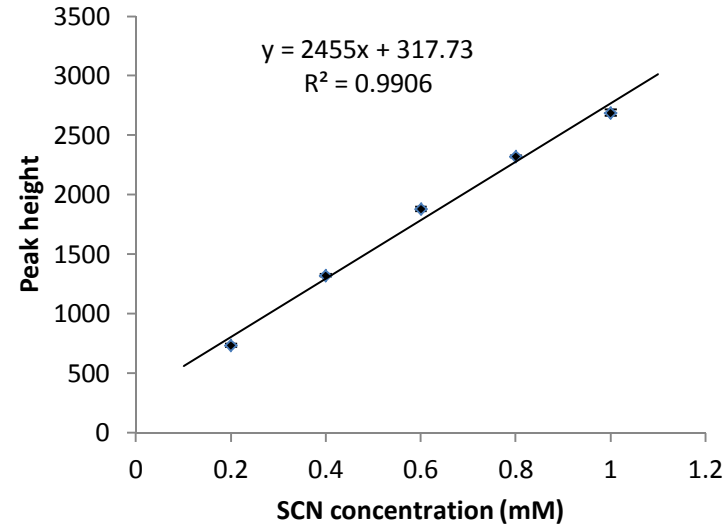


Figure 3. CE detection of thiocyanate and thiosulfate



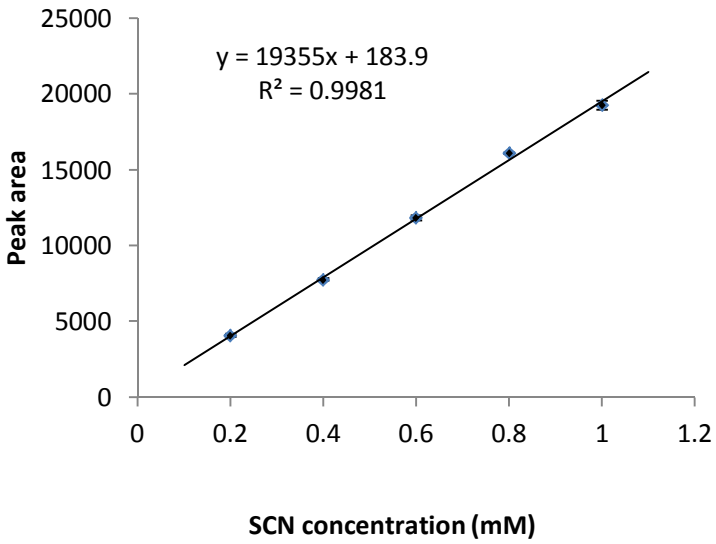


Figure 4. Standard SCN^- plot based on CE peaks at optimized condition. Each data point represents the average of ten injections with standard deviation.

3.2. Analysis of cyanide in cigarette smoke

CN in cigarette smoke extraction was converted into SCN^- by chemical reaction showing in Eq. 2 which was detected by CE. Fig. 5 shows a CE detection of SCN^- in the reaction product. To estimate whether pH of medium effects on the amount of CN extracted, smoke was extracted by 4 different isotonic solutions: NaCl 0.9% (pH 7.0), Dulbecco's phosphate buffered saline (PBS) (pH 7.4), NaCl 0.9% in HCl 10 mM (pH 2.0) and NaCl 0.9% in NaOH 10 mM (pH 12.0). The change of pH of the extraction was observed (Tab. 6). The analyzing the CE peak showed that level of dissolved CN was from 602 to 620 μM (24.08 to 24.80 μg/cigarette) with RSD lower than 3.27% for all extractions.

$$CN^- + H_2O_2 + S_2O_3^{2-} \rightarrow SCN^- + SO_4^{2-} + H_2O \quad (2)$$

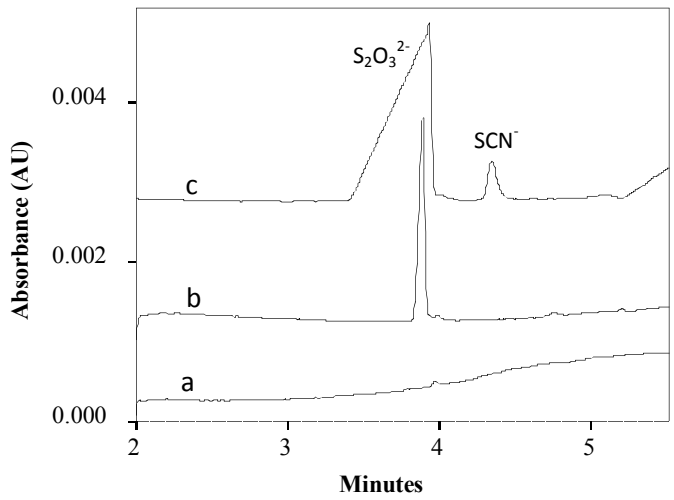


Figure 5. CE detection of SCN^- in reaction of H_2O_2 on CN in cigarette smoke. Trace arrangement is: (a): extraction of cigarette smoke; (b): 0.5 mM mixture of $S_2O_3^{2-}$ and H_2O_2; (c): mixture of (a) and (b) after 30 minutes incubating at 50^0C. CE conditions are summarized in section 2.2.

3.3. Detection of CN to SCN conversion in saliva and nasal discharge

Since the presence of CN in cigarette smoke was confirmed, the remaining question here is whether saliva can convert CN into SCN. To test this, we checked the presence of SCN in saliva and detected the SCN formed in the saliva after exposing to CN. The same analytical conditions were applied for nasal discharge. Level of SCN^- in saliva of smokers and non-smokers was first examined by CE detection. A result shown in Fig. 6 confirms the higher level of SCN^- in saliva of smokers than non-smokers. For this experiment, saliva and nasal discharge from smokers and non-smokers (10 each) were tested.

The formation of SCN^- in saliva and nasal discharge after treated with NaCN was detected by CE. Fig. 7A and 7B show the electropherograms obtained. No change in the peak intensity of SCN^- in the saliva or nasal discharge was found when compared to untreated ones (Fig. 7C), indicating no enzyme conversion of CN into SCN in saliva or nasal discharge.

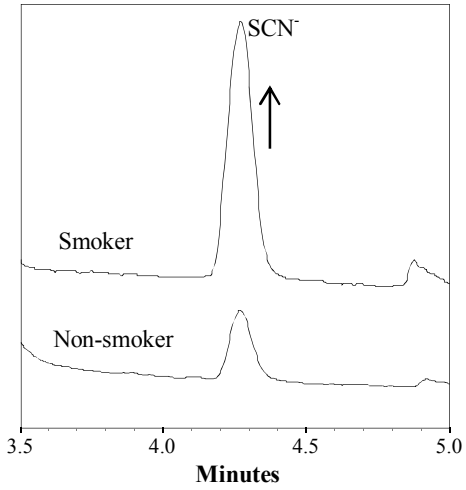


Figure 6. CE detection of SCN^- in saliva collected from smoker and non-smoker

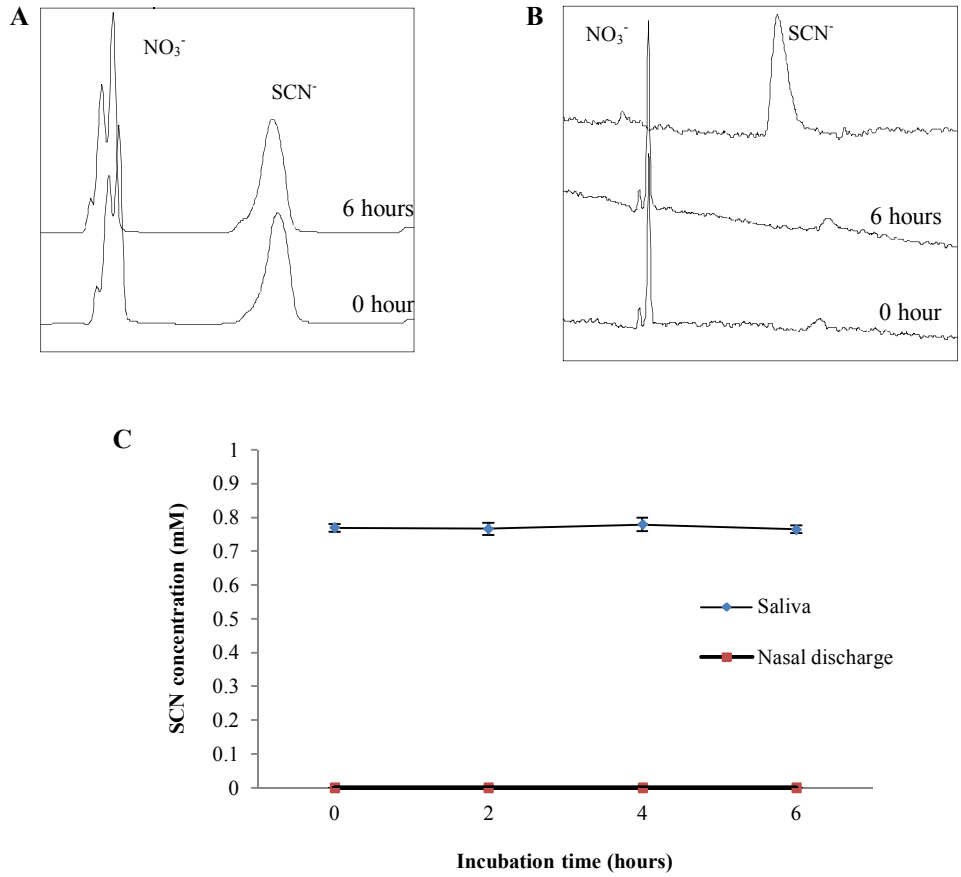


Figure 7. CE detection of SCN^- in cyanide exposed saliva (A) and nasal discharge (B) and correlative concentration (C) after 6 h at 37^0C.

3.4. Detection of CN to SCN conversion in lysates of oral cells

In the presence of $S_2O_3^{2-}$, CN^- was incubated with oral cell lysates prepared to estimate the conversion of CN into SCN. The formation of SCN^- was examined by colorimetric assay and CE. The CE data shown in Fig. 8A was obtained by using final concentration of 10 μg/ml of total protein. The chart in Fig. 8B was achieved using concentrations of 400 and 800 μg/ml of total protein. SCN^- was detected from both analytical methods, indicating that cell lysates converted CN into SCN. Among the tested cells, the highest activity was detected from YD-38 cells with higher SCN^- signal than others. This cell line was used for next experiment of the response of live cell on CN exposure.

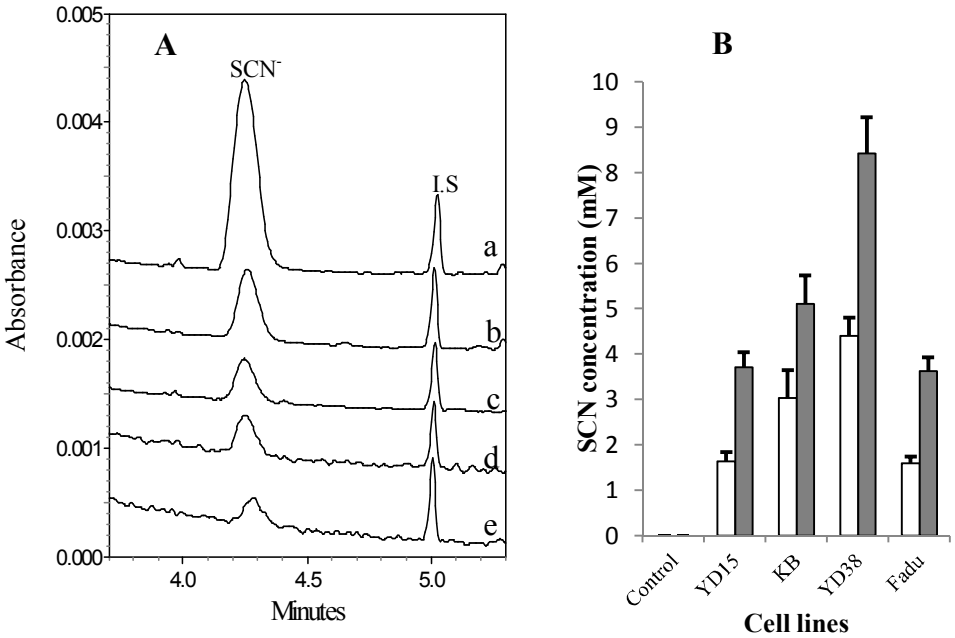


Figure 8. Detection and measurement of SCN^- after the treatment of cyanide on oral cell lysates. CE electropherograms of cyanide-treated on lysates (A) of fadu cells (e); YD-15 cells (d); KB cells (c); YD-38 cells (b) at concentration of 10 μg/ml of total protein are showed with 1 mM of SCN^- sample (a) is used as standard and 0.1 mM of urate sample is used as internal standard (I.S). The measurement of SCN^- concentration using colorimetric assay based on measuring of deep red complex of iron(III)-SCN is shown (B). The concentrations that were determined as level of total protein are 400 μg/ml (unfilled columns) and 800 μg/ml (filled columns).

3.5. Detection of rhodanese gene by RT-PCR

Since CN to SCN conversion was confirmed for cell lysates, cells were further probed for the conversion enzyme, rhodanese mRNA. RNAs from YD-15; YD-38; KB and fadu cells were isolated for RT-PCR. The PCR results showed the bands of the expected sizes (Fig. 9). Primer pairs of exon2F - exon2R bound to Rh exon2 region, exon3F - exon3R bound to Rh exon3 region, producing 520 bp and 347 bp in length, respectively. Between exon2 and exon3 is intron1 region with 6812 bp in length. Primer pairs exon2F - exon3R bind to cDNA template of Rh gene sequence (mRNA reference sequences: NM_001270483 and NM_003312) to make 983 bp in length of PCR product. Sequence analysis of these bands showed that rhodanese is expressed in oral cavity cells.

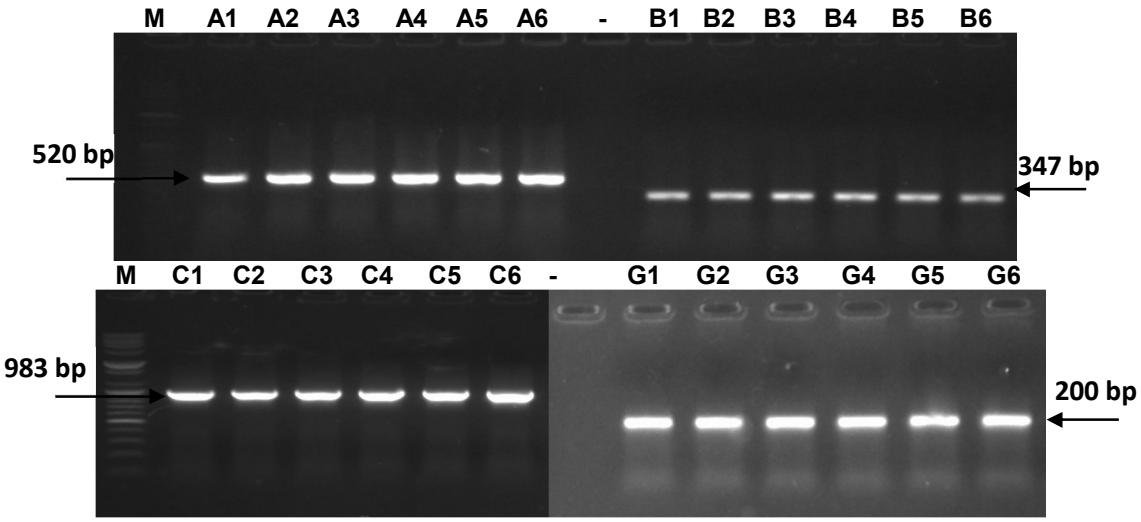


Figure 9. RT-PCR detection of rhodanese gene expression in oral cells. Total RNA from oral cells was isolated and reverse transcribed. PCR was performed using human oligo $(dT)_{12\text{-}18}$ primer. Bands of expected sizes including 520 bp for exon2F-exon2R (lane A), 347 bp for exon3F-exon3R (lane B), 983 bp for exon2F-exon3R (lane C) and 200 bp for GAPDH were seen. Markers (lane M) were 100 bp DNA ladder.

3.6. Detection of thiocyanate in cyanide exposed cells

Detecting SCN^- in culture media after cyanide exposure demonstrated the secretion of SCN^- from the cells. YD-38 cell line was chosen because its lysate showed the highest activity in CN to SCN conversion. The CE results in Fig. 11A showed that the level of SCN^- in culture medium was proportionally increased to the time of exposure. After 24 hours, the concentration of SCN^- was increased from 0 to 162.77 μM as summarized in Fig. 11B. These results supported that SCN formed inside cells was secreted out into culture environment.

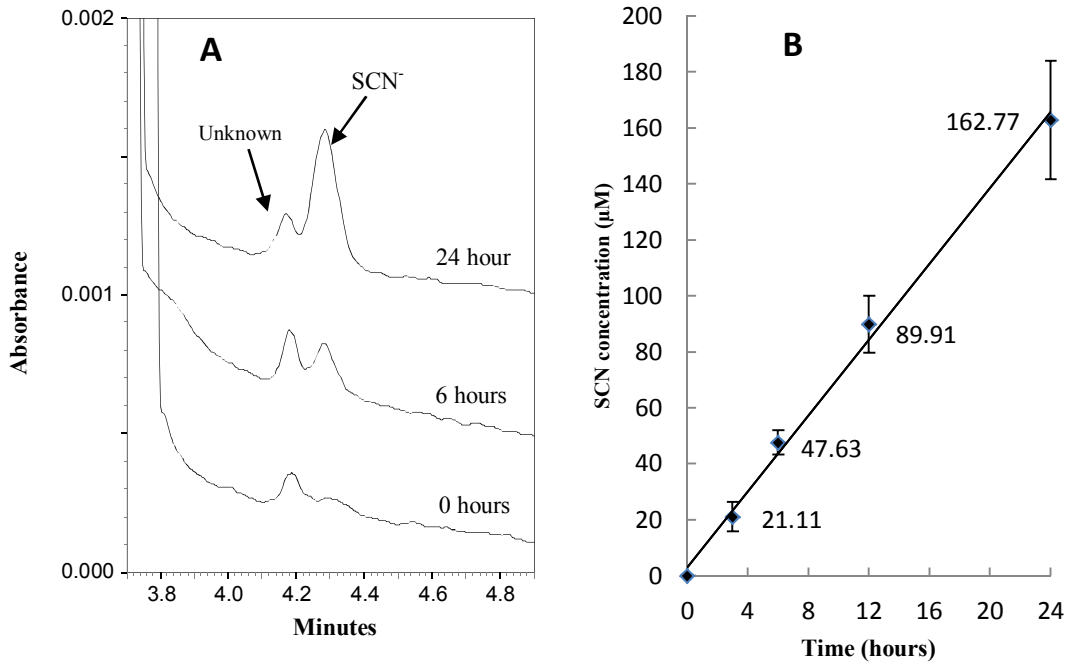


Figure 10. The CE detection of SCN^- in cyanide exposed YD-38 cell culture media. The electropherogram detection peak of SCN^- during 24 hrs of incubation at 37^0C is shown (A) and the increasing curve was drawn based on the analyzed peak area (B).

4. Discussion

The high concentration of SCN^- in saliva of smokers is due to the conversion of CN to SCN^- in cigarette smoke. However, the underlying roles of oral cavity on this conversion have not been documented. The question here was whether SCN^- in saliva was converted in oral cavity or not. In this thesis, we, therefore, confirmed the presence of CN in cigarette smoke and investigated the CN to SCN conversion in oral cavity *in vitro*.

Cigarette smoke contains more than 5000 chemical compounds. Many of them are known to be chemically reactive. The reported methods for analysis of CN in cigarette smoke are potentiometry, amperometry, fluorescence spectrometry, ion chromatography, spectrophotometry and segmented flow injection analysis [29]. In these methods, common problems were the use of carcinogenic or mephitic reagents that pollute the environment and the determination was commonly interfered by other ions. Furthermore, the procedures were complicated and time-consumed. Interestingly, the conversion of CN to SCN in the presence of thiosulfate and hydrogen peroxide was not only selective but also UV-active SCN was fast detected by CE.

Regarding anion detection by CE, the main parameter induced the migration time of anion is the mobility of electro osmotic flow (EOF) because the EOF is directed toward the negative charge of cathode, opposite direction to the anion migration. Thus, the velocity of an anion analyte inside capillary depends upon the rate of EOF of the buffer solution. In other words, reducing EOF mobility provides faster analysis. Since the EOF is significantly depend on the negative charge of silanol (Si-OH) groups in

fused-silica capillary, adding cationic surfactants or alkylamines tends to neutralize the negative charge on surface of the fused silica capillary, thus allow to control the EOF mobility and analysis time as well [30]. These compounds are often called osmotic flow modifiers (OFM). In the present work, we obtained the fast analysis of SCN^- and $S_2O_3^{2-}$ by using 1mM of myristyltrimethylammonium hydroxide as an OFM. The presence of OFM in BGE reduced the migration time of both analytes by more than 60 % without affecting the resolution.

The data suggested that even exposed to high concentration of CN, saliva or nasal discharge were not able to convert CN into SCN. This is due to the absence of rhodanese enzyme in fluids.

Cyanide treatment alkalified the culture medium and affected on cell growth. The medium after treating with 25 mM NaCN became pH 9.0 and induced necrotic cell death. Hence, addition of buffer into culture medium was therefore critical to maintain the experiment condition by reducing the pH change. In this experiment, 25 mM HEPES was used to keep the pH value in a range from 7.2 to 7.6 during the treatment of 1; 5; 10 and 25 mM CN. Those pHs maintained cells in good condition for up to 48 h.

Together with RT-PCR result, the confirmation of conversion activity in cell lysates supports the presence of rhodanese in oral cells. Interestingly, the detection of SCN^- formation from cells exposed to CN suggests that cells can convert CN into SCN inside and secret out into culture media. In conclusion, oral cells are the source of rhodanese enzyme. They response to the uptaken CN by conversion into SCN^- and provide SCN^- in saliva (Fig. 11).

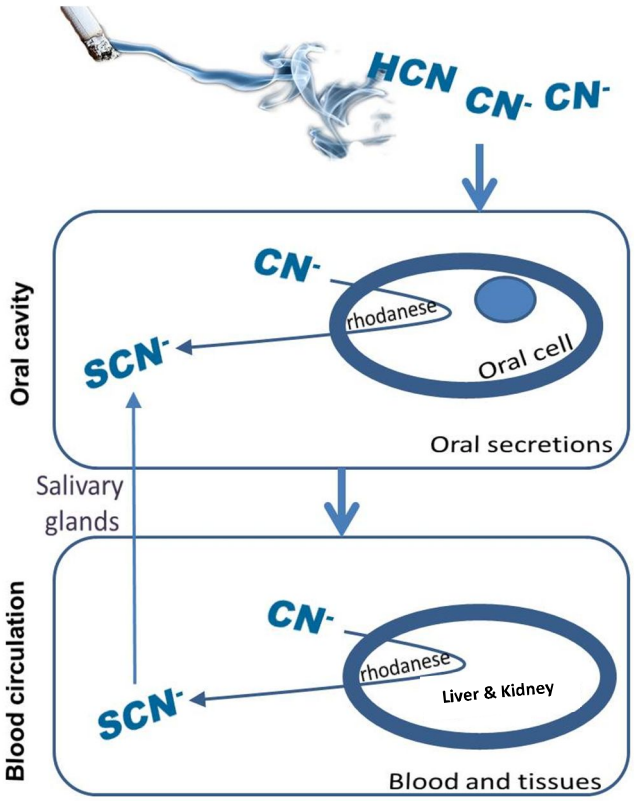


Figure 11. The generation and accumulation of SCN^- in saliva by rhodanese-catalyzed CN intoxication. Primary source of SCN is occurred within oral cells and secondary source is secreted from saliva glands after generating in other tissues.

5. Conclusions

A CE method was successfully applied for the qualification of CN substance in cigarette smoke as well as characterizing the conversion of CN into SCN *in vitro*. Our experimental results demonstrated that the CN to SCN conversion that has been believed to mainly happen in liver and kidney also occurs within oral cavity. Saliva and nasal discharge did not convert CN into SCN. Oral cancer cell lines (YD-15, YD-38, KB and fadu) were confirmed to express the conversion enzyme, rhodanse, gene and detect of CN to SCN conversion by cells. These data suggests that oral cells contains rhodanese which convert CN into SCN and provide source of SCN in saliva.

6. References

1. Alcorta R,FACEP M. Smoke inhalation & acute cyanide poisoning. Hydrogen cyanide poisoning proves increasingly common in smoke-inhalation victims. JEMS. 2004;29:6-15.
2. Norman V, Ihrig A, Larson T, Moss B. The effect of some nitrogenous blend components on no/nox and hcn levels in mainstream and sidestream smoke. Beiträge zur Tabakforschung/Contributions to Tobacco Research. 1983;12:55-62.
3. Fiksel J. Quantitative risk analysis for toxic chemicals in the environment. Journal of hazardous materials. 1985;10:227-240.
4. Blanc P, Hogan M, Mallin K, Hryhorczuk D, Hessl S, Bernard B. Cyanide intoxication among silver-reclaiming workers. JAMA. 1985;253:367-371.
5. El Ghawabi SH, Gaafar MA, El-Saharti AA, Ahmed SH, Malash KK, Fares R. Chronic cyanide exposure: A clinical, radioisotope, and laboratory study. British Journal of Industrial Medicine. 1975;32:215-219.
6. Guidon G. Boisclaird. Past, current and future trends in tobacco use. Health nutrition and population discussion paper, World Bank Human Development Network. 2003;
7. Saidu Y. Physicochemical features of rhodanese: A review. African Journal of Biotechnology. 2005;3:370-374.
8. BONOMI F, PAGANI S, CERLETTI P, CANNELLA C. Rhodanese-mediated sulfur transfer to succinate dehydrogenase. European Journal of Biochemistry. 1977;72:17-24.
9. Cerletti P. Seeking a better job for an under-employed enzyme: Rhodanese. Trends in Biochemical Sciences. 1986;11:369-372.

10. Pagani S, Bonomi F, Cerletti P. Sulfide insertion into spinach ferredoxin by rhodanese. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology. 1982;700:154-164.
11. Ogata K, Dai X, Volini M. Bovine mitochondrial rhodanese is a phosphoprotein. Journal of Biological Chemistry. 1989;264:2718-2725.
12. Ogata K,Volini M. Mitochondrial rhodanese: Membrane-bound and complexed activity. Journal of Biological Chemistry. 1990;265:8087-8093.
13. Nandi DL, Horowitz PM, Westley J. Rhodanese as a thioredoxin oxidase. The international journal of biochemistry & cell biology. 2000;32:465-473.
14. Aminlari M,Gilanpour H. Comparative studies on the distribution of rhodanese in different tissues of domestic animals. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry. 1991;99:673-677.
15. Aminlari M, Malekhusseini A, Akrami F, Ebrahimnejad H. Cyanide-metabolizing enzyme rhodanese in human tissues: Comparison with domestic animals. Comparative Clinical Pathology. 2007;16:47-51.
16. Fragoso MA, Fernandez V, Forteza R, Randell SH, Salathe M, Conner GE. Transcellular thiocyanate transport by human airway epithelia. The Journal of physiology. 2004;561:183-194.
17. Baskin SI,Kirby SD. The effect of sodium tetrathionate on cyanide conversion to thiocyanate by enzymatic and non-enzymatic mechanisms. Journal of Applied Toxicology. 1990;10:379-382.
18. Sörbo B. [43] rhodanese: Cn−+ s2o3−−→ cns−+ so3−−. Methods in enzymology. 1955;2:334-337.

19. Fukushi K, Takeda S, Chayama K, Wakida S-I. Application of capillary electrophoresis to the analysis of inorganic ions in environmental samples. Journal of Chromatography A. 1999;834:349-362.
20. Aminlari M,Vaseghi T. Biochemical properties and biological functions of the enzyme rhodanese in domestic animals. Iranian Journal of Veterinary Research. 2006;7:1-13.
21. Dacre JC,Tabershaw IR. Thiocyanate in saliva and sputum: Relationship to smoking and industrial exposures. Archives of Environmental Health: An International Journal. 1970;21:47-49.
22. Schultz CP, Ahmed MK, Dawes C, Mantsch HH. Thiocyanate levels in human saliva: Quantitation by fourier transform infrared spectroscopy. Analytical biochemistry. 1996;240:7-12.
23. Lorentzen D, Durairaj L, Pezzulo AA, Nakano Y, Launspach J, Stoltz DA, Zamba G, McCray PB, Zabner J, Welsh MJ. Concentration of the antibacterial precursor thiocyanate in cystic fibrosis airway secretions. Free Radical Biology and Medicine. 2011;50:1144-1150.
24. Lundquist P, Mårtensson J, Sörbo B, Ohman S. Method for determining thiocyanate in serum and urine. Clinical chemistry. 1979;25:678-681.
25. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry. 1976;72:248-254.
26. Agboola FK,Okonji RE. Presence of rhodanese in the cytosolic fraction of the fruit bat (eidolon helvum) liver. BMB Reports. 2004;37:275-281.
27. Rozen S,Skaletsky H. Primer3 on the www for general users and for biologist programmers. Bioinformatics methods and protocols. 1999;365-386.

28. Nonis A, Scortegagna M, Nonis A, Ruperti B. Prato: A web-tool to select optimal primer pairs for qpcr. Biochemical and biophysical research communications. 2011;415:707-708.
29. Jiang Y, Lu N, Yu F, Li Q, Xu H. Sampling and determination of hydrogen cyanide in cigarette smoke. Fresenius' journal of analytical chemistry. 1999;364:786-787.
30. Sladkov V,Fourest B. Separation and determination of some carboxylic acids by capillary electrophoresis. 2006;